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The curious world of apoptotic cell clearance

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Analysis of knockout mice has brought into question the previously proposed role of the phosphatidylserine receptor (Ptdsr) in the clearance of apoptotic cell corpses, and has suggested important functions in regulating differentiation and inflammation.

"Curiouser and curiouser!" cried Alice when she realized the startling effects of ingesting a small cake on which the words 'Eat me' were beautifully marked in currants [1]. The world of apoptosis research is every bit as wonderful and full of surprises as the Wonderland that Alice discovered. Dying cells display enticing 'eat me' signals and a collection of colorful molecular characters to ensure their digestion. Now, in *Journal of Biology* [2], Andreas Lengeling and colleagues reveal more surprises about the phosphatidylserine receptor (Ptdsr) molecule that was first cloned as a receptor responsible for the phosphatidylserine-specific clearance of apoptotic cells (see 'The bottom line' box for a summary of the work).

Body snatching

Large numbers of cells die by **apoptosis** during the development of multicellular organisms (see the 'Background' box), and many research groups are hunting down the molecular culprits responsible for clearing up the corpses. Apoptotic cells are removed by a process involving recognition and **phagocytosis**, followed by the induction of an active anti-inflammatory response. These events are critical for efficient corpse elimination and to prevent the leakage of potentially cytotoxic or antigenic cellular contents that could elicit an autoimmune response; defects in apoptotic cell clearance are associated with autoimmune and inflammatory diseases.

In order to be recognized for removal, dying cells present signals at the cell surface that trigger engulfment either by professional phagocytes

The bottom line

- The gene encoding the phosphatidylserine receptor (*Ptdsr*) was originally cloned as the antigen recognized by a monoclonal antibody that prevents macrophages from engulfing dying cells and removing apoptotic corpses.
- The gene has now been inactivated in mice in three laboratories independently, to examine its role in apoptotic cell clearance and anti-inflammatory signaling.
- The newest strain of *Ptdsr*-deficient mice died around birth and showed dramatic defects in the development of many tissues including lungs, kidneys, intestines, and eyes.
- The engulfment and removal of apoptotic cells appears not to be affected in these *Ptdsr*-knockout mice, but production of cytokines is impaired by *Ptdsr*-deficient macrophages that regulate inflammation.
- It seems that Ptdsr is not required for the clearance of apoptotic cells but plays unexpected roles, controlling cell differentiation during development and cytokine production by macrophages.

Background

- Cells that die by the suicide program called **apoptosis** are phagocytosed - engulfed and digested by nearby cells - to prevent harmful leakage of cellular contents. Apoptosis and clearance of dying cells are essential for normal development.
- **Phagocytosis** is induced by 'eat me' signals expressed on the apoptotic cell surface that are recognized by receptors on adjacent cells. The phospholipid **phosphatidylserine** (**PS**) is proposed to be a primary 'eat me' signal; it is exposed only on the surface of dying cells.
- **Phage display** can be used to screen a library of recombinant peptides expressed on the surface of bacteriophages, and was used to identify the antigen recognized by a phagocytosis-inhibiting monoclonal antibody mAb 217 during the cloning of the phosphatidylserine receptor Ptdsr.
- The **genetic background** of inbred mouse strains can have a severe (and unpredictable) effect on the phenotypes of knockout strains. Commonly used strains, such as C57BL/6J (a black mouse) and 129 (an agouti brown mouse), have known and unknown differences at numerous alleles (see Figure 1).

(macrophages) or by amateurs (neighboring cells). The best known of these signals is the phospholipid **phosphatidylserine** (**PS**) [3]. A large number of proteins have been reported to bind to exposed PS molecules on dying cells; some bind to PS directly and some via bridging molecules. Working out why there are so many PS-binding proteins and how they all work is a major preoccupation of apoptosis researchers.

A few years ago, Valerie Fadok, Peter Henson and colleagues, at the National Jewish Medical and Research Center in Denver, Colorado, generated monoclonal antibodies that prevent phagocytosis by human macrophages [4]. The antibodies also stimulated the production of transforming growth factor-β (TGF-β) and blocked the production of the inflammatory cytokine tumor necrosis factor-α (TNF-α), suggesting a link between PS recognition and downregulation of the inflammatory response after the uptake of apoptotic cells. Henson's group used **phage display** to clone the antigen recognized by one of these antibodies, mAb 217 [4]. They reported that the mAb 217 recognized a predicted transmembrane PS receptor that was similar to homologs in *Caenorhabditis elegans* and *Drosophila melanogaster*, suggesting conservation of function. Henson and colleagues proposed this gene as a good candidate for a PS-specific receptor that is critical for mediating the uptake of apoptotic cells, adding a cautionary note that "we cannot rule out at this time that it facilitates PS recognition by some other function which does not involve direct binding to PS" [4].

At around the same time, Lengeling was setting up his group at the German Research Center for Biotechnology (GBF) in Braunschweig, Germany. The GBF has a focus on infectious diseases, and Lengeling was interested in phagocytosis by macrophages in different mouse models (see the 'Behind the scenes' box for more of the rationale for the work). The Fadok and Henson paper excited Lengeling, who saw parallels between the recognition of pathogens and the recognition of apoptotic cells. "In both cases the phagocytes make use of germline-encoded receptors," he notes. "The difference is that phagocytes recognize 'self' antigen molecules on apoptotic cells instead of the 'foreign' molecules presented by pathogens. But most importantly, the reaction of a macrophage is completely different if it sees apoptotic cells or a pathogen; pathogens trigger pro-inflammatory reactions, whereas apoptotic cells induce strong anti-inflammatory reactions."

The PS receptor knockouts

Lengeling's group was not the only one keen to figure out what the PS receptor does: at least two other groups were also generating and characterizing a mouse knockout for the PS receptor (Ptdsr) [5,6]. But comparison of the reports from the different groups is puzzling. Li *et al.* [5] concluded that the PS receptor is essential for the removal of apoptotic cells and saw defective phagocytosis of apoptotic cells by macrophages derived from their *Ptdsr*-knockout mice. They also found an accumulation of dead cells in the lung and brain, which they suggested could explain the observed neonatal lethality. A few months later, Kunisaki *et al.* [6] reported that erythropoiesis and T-cell lymphopoiesis were blocked in their *Ptdsr*-knockout mouse strain, and that this mutation also resulted in impaired clearance of apoptotic cells in the liver and thymus.

Lengeling's group was bemused; they could find no evidence for impaired clearance using several different techniques, both *in vitro* and *in vivo*, looking at different organs and developmental stages. But their mice had so many other phenotypes that they had their hands full [2]. There was severe perinatal lethality and a large number of defects in different tissues, all of which were related to delayed

differentiation. Embryos were growth-retarded, with malformations of the head, palate, and the developing eye. The Ptdsr-deficient embryos also had a delay in tissue differentiation in the lung, kidney, and intestine. Brain hyperplasia and a block in erythropoietic differentiation were also observed, as in the reports from Li *et al.* [5] and Kunisaki *et al.* [6], respectively. One of the most striking defects was the absence of eyes in some embryos associated with the induction of ectopic eye structures in nasal cavities. Finally, they observed reduction in the levels of macrophage cytokines that had not been reported by the other groups.

Reconciling the results

The existence of several knockouts of the same gene with very different phenotypes is puzzling, intriguing, and divides researchers in the field about how to interpret the results. Simon Brown of the University of Edinburgh, UK, urges readers to focus on the commonality of the three studies. "All three found the homozygous-null mouse to be perinatal lethal with clear evidence of a defect in cell differentiation and marked effects on tissue and organ development following the mid-gestation period," he notes. He admits that there are some major differences that draw one's attention but suspects these can be explained by differences in experimental approaches. Most researchers seem to agree that Lengeling's analysis is particularly careful, but the discrepancies between the different studies remain perplexing.

Michael Hengartner at the University of Zurich, Switzerland, is unequivocal. "Lengeling's results clearly demonstrate that Ptdsr is most certainly not a PS receptor, and probably has nothing to do with apoptotic cell recognition at all." He suspects that the source of the problem is a case of false identification during the expression-cloning of the antigen recognized by mAb 217. This is supported by the supplementary data provided by Böse *et al.* [2], in which mAb 217 is shown to recognize macrophages derived from their knockout mouse [2]. "One wonders why the other two groups did not perform this control using their mice," notes Hengartner. Shigekazu Nagata from the Osaka University Medical School, Japan, also feels that the cloned *Ptdsr* gene had not been sufficiently characterized previously. "Lengeling's group now shows that Ptdsr carries an epitope that can be weakly recognized by the mAb 217. But the antibody efficiently stains even the Ptdsr-deficient cells, indicating that the antibody recognizes a molecule other than Ptdsr."

"The PS receptor story is an interesting case of how an excusable error, that can easily happen in any scientific pursuit, results in a series of published data that are guided by prejudice," says Angelika Böttger from the Ludwig-Maximilians-Universität in Munich, Germany. She is sure that the mAb 217 antibody really does inhibit the phagocytosis of apoptotic cells. "The only problem is that the Ptdsr gene does not encode the antigen for this antibody." She says that the experiment in which the Ptdsr-deficient cells are stained with the mAb 217 "should finally convince everybody that the dogma is wrong."

Other researchers remain unconvinced. "One should not rush to conclude that Ptdsr is not important for corpse removal based on the analysis of one mouse *Ptdsr*-knockout line," cautions Ding Xue from the University of Colorado in Boulder. "The differences in mutant phenotypes observed in the three different mouse lines, including apoptotic corpse removal, are likely due to the different **genetic backgrounds** of the knockout mice or differences in carrying out various assays," he says. "I think that the Lengeling group should at least analyze the mouse line from Li *et al.* or Kunisaki *et al.* before making any definitive conclusions." Xue cites numerous precedents in which different genetic backgrounds yield dramatically different mutant phenotypes "For example, caspase3-deficient mice in a C57BL/6J background are viable, but are nonviable in a 129 background. In such circumstances, one needs to be cautious about stating whether the results obtained from one mouse line are more reliable or credible than the other lines: most likely, the results from three groups were all correct in respect to the mouse lines that they examined." Lengeling's group used an isogenic C57BL/6J background, whereas the previous *Ptdsr* knockouts were in a mixed 129 x C57BL/6 background (see Figure 1).

Figure 1
Genetic background variation in mouse strains, as shown by two adult mice with their pups. The C57BL/6J mouse (black coat) was crossed with a chimeric mouse (patchy coat), consisting of mutant C57BL/6J cells in a BALB/c white background. The offspring with a black coat color can then be screened for germline transmission of the mutant allele. Image: Ozgene Pty. Ltd.

Siamon Gordon, a macrophage expert at the University of Oxford, UK, feels that "the main point of this article is that people were not looking hard enough before and were jumping to conclusions." But Henson himself welcomes the different results. "The more

Behind the scenes

Journal of Biology asked Andreas Lengeling about the background and rationale for his study of the phosphatidylserine receptor (*Ptdsr*) in mice.

What motivated you to generate a *Ptdsr* knockout mouse?
My group is interested in the function of macrophages in immune responses and how they defend the body during infection. We were fascinated by the emerging work on Toll receptors in innate immunity and the recognition of pathogens. Valerie Fadok's work introduced the scientific community to a new receptor, Ptdsr, which could specifically recognize host apoptotic cells, and exposed PS as a key signal for phagocyte engulfment. Ptdsr seemed to be crucial for two kinds of macrophage responses: the engulfment of apoptotic cells and the release of immunosuppressive mediators. We knocked out the gene encoding Ptdsr to examine its role in these processes.

How long did the experiments take and what were the steps that ensured success?
Knocking out the *Ptdsr* gene turned out not to be too difficult, thanks to Frank Köntgen at Ozgene. The analysis of the mutant mice turned out to be more complicated, especially because ablation of *Ptdsr* was lethal. We looked hard for differences in the efficacy of apoptotic cell removal in mutant animals but couldn't see any. Instead we identified a lot of interesting phenotypes, which pointed us towards completely novel and unexpected functions of Ptdsr during development. This success was the work of a team of gifted specialists, including veterinary pathologist Achim Gruber.

What was your initial reaction to the results and how were they received by others?
At first we had a hard time believing that there was actually no impairment in apoptotic cell removal in our knockout mice. Eventually we realized that Ptdsr functions as a differentiation-promoting factor in many different organs and tissues during embryogenesis. Surprisingly, this was received quite openly in the community. It turned out that scientists working in other model systems, such as flies, worms, and even *Hydra*, also had evidence for alternative Ptdsr functions.

What are the next steps?
There are two major things that need to be followed up. First, as Ptdsr is not the major receptor for apoptotic cells, the question remains whether there is a specific PS receptor out there or whether PS is only recognized by 'bridging molecules'. This will require elegant interdisciplinary approaches that compare different animal model systems, such as mice, flies, and worms. Second, we want to investigate the primary function of Ptdsr by generating conditional alleles of *Ptdsr*, by investigating downstream pathways via gene-expression array profiling, and by doing a lot of biochemistry.

studies we have on this molecule the more interesting it gets, and it clearly has multiple functions." Henson confides that his group has generated a fourth knockout strain and preliminary results suggest that the phenotypes differ from the other three. He admits that it appears confusing, but is confident that the data will eventually fit together." Gordon suggests that different cells may contribute to clearance in different scenarios. "Macrophages are faster and more efficient professional phagocytes than non-leukocytes, so they may be the main players during inflammation or infection." He notes that earlier studies had indicated that macrophages are not essential for apoptotic clearance during development, adding that *C. elegans* has no macrophages but can still clear corpses.

More surprises in store

Many experts hope that clues will come from analysis of the Ptdsr protein in other species. Xue's group has analyzed the role of the *Ptdsr* gene in worms and found evidence that it is involved in removing apoptotic cells [7]. But Kristin White's group at MGH-Harvard in Charlestown, USA, has studied the PS receptor in flies and came up with results that fit more with those in the Lengeling article. "We see no obvious defect in engulfment of apoptotic cells in *Drosophila* embryos that lack the PS receptor. These animals are viable, with some subtle developmental defects," says White.

Future studies will obviously focus on functions of the Ptdsr beyond apoptotic cell clearance. Nagata hopes that the Lengeling study will trigger investigation of the 'real' function of Ptdsr during mammalian development. "These have little in common; thus Ptdsr probably has a specific cellular role that is required at multiple occasions throughout development," says Hengartner. White agrees: "Since we also see effects on development in the fly PS receptor mutant, this suggests that the PS receptor has a biological

function that is more general than the recognition of apoptotic cells. The use of different biological systems and approaches to dissect the role of this protein should help us to understand this more general role."

There are likely to be more surprises in store. "The Ptdsr protein carries a domain called the Jumonji C (JmjC) domain," notes Nagata, speculating that, like other proteins with this domain, Ptdsr may play a role in chromatin remodeling and the stability of heterochromatin. Edinburgh's Brown makes similar predictions and indeed, there is evidence that the Ptdsr protein may be located in the nucleus [8,9]. Böttger, whose lab studies the PS receptor from *Hydra*, suggests "it could modify nuclear proteins, transcription factors or maybe proteins maintaining nuclear architecture and thus have these profound effects on differentiation during early mouse development."

Alice's adventures came to an end when she awoke from her dream. But our apoptotic adventures are likely to continue for some time as we learn more about the surprises that govern when and how cells die and who is responsible for clearing up the remains.

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